Profiling Serine Hydrolase Activities in Complex Proteomes†

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ABSTRACT: Serine hydrolases represent one of the largest and most diverse families of enzymes in higher eukaryotes, comprising numerous proteases, lipases, esterases, and amidases. The activities of many serine hydrolases are tightly regulated by posttranslational mechanisms, limiting the suitability of standard genomics and proteomics methods for the functional characterization of these enzymes. To facilitate the global analysis of serine hydrolase activities in complex proteomes, a biotinylated fluorophosphonate (FP-biotin) was recently synthesized and shown to serve as an activity-based probe for several members of this enzyme family. However, the extent to which FP-biotin reacts with the complete repertoire of active serine hydrolases present in a given proteome remains largely unexplored. Herein, we describe the synthesis and utility of a variant of FP-biotin in which the agent's hydrophobic alkyl chain linker was replaced by a more hydrophilic poly(ethylene glycol) moiety (FP-peg-biotin). When incubated with both soluble and membrane proteomes for extended reaction times, FP-biotin and FP-peg-biotin generated similar "maximal coverage" serine hydrolase activity profiles. However, kinetic analyses revealed that several serine hydrolases reacted at different rates with each FP agent. These rate differences were exploited in studies that used the biotinylated FPs to examine the target selectivity of reversible serine hydrolase inhibitors directly in complex proteomes. Finally, a general method for the avidin-based affinity isolation of FP-biotinylated proteins was developed, permitting the rapid and simultaneous identification of multiple serine peptidases, lipases, and esterases. Collectively, these studies demonstrate that chemical probes such as the biotinylated FPs can greatly accelerate both the functional characterization and molecular identification of active enzymes in complex proteomes.

A major focus of post-genomic research is the development of methods and technologies that permit the integrated analysis of numerous biomolecules in samples of high complexity. These global experimental approaches include: (i) genomics or the comparative analysis of the mRNA expression patterns (transcriptomes) of cells/tissues (*1*, *2*), and (ii) proteomics or the comparative analysis of the protein expression patterns (proteomes) of cells/tissues/fluids (*3*, *4*). It is anticipated that the effective application of genomics and proteomics methods will accelerate the functional characterization of genes and gene products, thereby facilitating our understanding of the molecular basis of health and disease. Notably, several recent studies employing gene chip technologies have demonstrated how transcript profiling can enable the classification of cancer subtypes (*5*, *6*) and the identification of gene products involved in oncogenesis (*7*). Still, the reliance of genomics methods on mRNA levels as readouts of protein expression and activity remains a matter of significant concern, especially in light of studies emphasizing the generally modest correlation that exists between transcript and protein levels (*8*, *9*).

Recently, efforts have emerged to analyze the proteome itself, most of which have utilized two-dimensional gel electrophoresis (2DE)[1] and protein staining/mass spectrometry as separation and detection methods, respectively (*10*). Significant advances have been made in the development of automated 2DE-MS systems for the rapid analysis of complex proteomes (*11*). Nonetheless, current proteomics endeavors still face formidable technical challenges, especially with regard to the limited separation potential of 2DE. Several types of proteins, including membrane-bound (*12*) and low abundance proteins (*13*), have proven difficult to analyze by 2DE. Additionally, 2DE-MS-based proteomics methods, by measuring changes in protein abundance, offer like genomics only an indirect readout of dynamics in protein activity. Both approaches fail to detect numerous posttranslational forms of protein regulation critical for controlling molecular and cellular function.

In efforts to address some of the shortcomings of standard genomics and proteomics approaches, we have initiated a research program aimed at developing chemical reagents that profile significant fractions of the proteome in an activity-dependent manner (*14*). We recently described the synthesis and utilization of a biotinylated fluorophosphonate (FP-

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[1] Abbreviations: FAAH, fatty acid amide hydrolase; FP, fluorophosphonate; MALDI, matrix assisted laser desorption; MS, mass spectrometry; OTFMK, oleoyl trifluoromethyl ketone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 2DE, two-dimensional page electrophoresis.

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biotin) as a prototype activity-based proteomics probe that targets the serine hydrolase family of enzymes (*15*). Serine hydrolases represent one of the largest and most diverse classes of enzymes in higher eukaryotes, collectively composing ~3% of the predicted *Drosophila* proteome (*16*). Subclasses of serine hydrolases include proteases, lipases, esterases, amidases, and transacylases, with individual enzymes implicated in numerous (patho)physiological processes, such as blood coagulation (*17*), T cell cytotoxicity (*18*), neural plasticity (*19*), neurotransmitter catabolism (*20*, *21*), peptide/protein processing (*22*), protein/lipid digestion (*23*), angiogenesis (*24*), emphysema (*25*), and cancer (*26*). Importantly, the activities of many serine hydrolases are regulated in a posttranslational manner [e.g., zymogen cleavage for catalytic activation (*27*), inhibitor binding for catalytic inactivation (*28*, *29*)], indicating that standard, abundance-based genomics and proteomics methods may be of limited use for the functional analysis of these enzymes.

As a result of a shared catalytic mechanism, nearly all serine hydrolases are irreversibly inhibited by FP-based reagents (*30*, *31*). Additionally, the reaction of FPs with serine hydrolases depends on these enzymes being catalytically active (*15*, *32*). Thus, in principle, FP-biotin could provide a complete profile of the active serine hydrolases present in a given proteome. However, the extent to which FP-biotin comprehensively reacts with all of the members of the serine hydrolase family remains unclear. Additionally, to be of lasting value to the field of proteomics, chemical probes such as FP-biotin should not only detect but also accelerate the molecular identification of active enzymes in samples of high complexity. Herein, we report a series of studies that explores in detail the reactivity of biotinylated FPs with serine hydrolases in complex proteomes, revealing that these agents have broad application to the functional analysis of this enzyme family. In particular, biotinylated FPs were used to (i) compare the expression of numerous serine hydrolase activities across both soluble and membrane proteomes, (ii) determine the target selectivity of reversible serine hydrolase inhibitors directly in complex proteomes, and (iii) isolate and molecularly characterize multiple serine hydrolases in a rapid and consolidated manner.

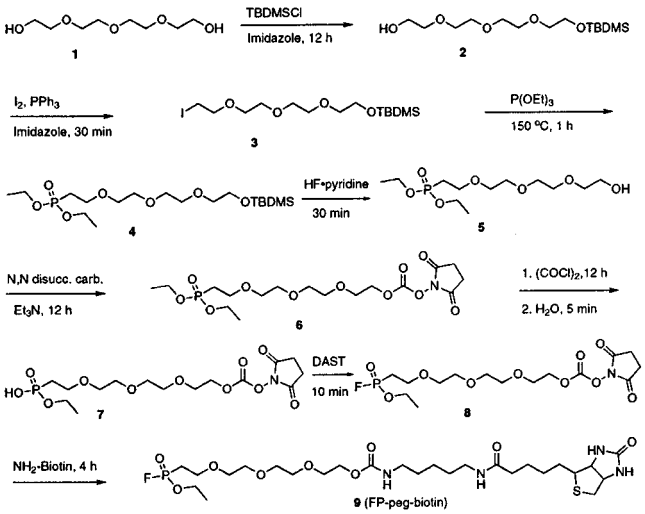
EXPERIMENTAL PROCEDURES

*Chemical Synthesis of FP-Peg-Biotin (**9**).* Compound numbers in bold refer to structures shown in Scheme 1.

*Compound **2**.* A solution of **1** (3.9 g, 20.0 mmol, 3.0 equiv) in DMF (8.0 mL) was treated with TBDMSCl (1.0 g, 6.7 mmol, 1.0 equiv) and imidazole (0.9 g, 13.3 mmol, 2.0 equiv), and the reaction mixture was stirred for 12 h at room temperature. The reaction mixture was then quenched with saturated aqueous $NaHCO_3$ and partitioned between ethyl acetate (200 mL) and water (200 mL). The organic layer was washed with saturated aqueous NaCl (100 mL), dried (Na_2SO_4), and concentrated under reduced pressure. Chromatography (SiO_2, 5 × 15 cm, 50−100% ethyl acetate−hexanes) afforded **2** (1.1 g, 2.0 g theoretical, 55%) as a colorless oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.8−3.5 (m, 16H, CH_2OR), 0.88 (s, 9H, CH_3C), 0.0 (s, 6H, CH_3Si); MALDI−FTMS *m*/*z* 331.1907 ($C_{14}H_{32}O_5Si + Na^+$ requires 331.1911).

*Compound **3**.* A solution of **2** (0.61 g, 2.0 mmol, 1.0 equiv) in benzene (15 mL, 0.13 M) was treated sequentially with

Scheme 1: Synthesis of FP-Peg-Biotin (**9**)



PPh_3 (2.6 g, 10.0 mmol, 5 equiv), I_2 (2.3 g, 9.0 mmol, 4.5 equiv), and imidazole (0.7 g, 10.3 mmol, 5.2 equiv), and the reaction mixture was stirred at room temperature for 30 min, producing a yellow-orange heterogeneous solution. The soluble portion of the reaction mixture was removed and the insoluble portion was washed several times with ethyl acetate. The combined reaction and washes were then partitioned between ethyl acetate (200 mL) and saturated aqueous $Na_2S_2O_3$ (200 mL). The organic layer was washed sequentially with H_2O (100 mL) and saturated aqueous NaCl (100 mL), dried (Na_2SO_4), and concentrated under reduced pressure. Chromatography (SiO_2, 5 × 15 cm, 5−25% ethyl acetate−hexanes) afforded **3** (0.54 g, 0.82 g theoretical, 66%) as a colorless oil: ^{1}H NMR ($CDCl_3$, 400 MHz) 3.85−3.60 (m, 12H, CH_2OR), 3.54 (t, J = 5.6 Hz, 2H, $CH_2OTBDMS$), 3.23 (t, J = 7.0 Hz, 2H, CH_2I), 0.88 (s, 9H, CH_3C), 0.0 (s, 6H, CH_3Si); ESI *m*/*z* 441 ($C_{14}H_{31}IO_4Si + Na^+$ requires 441).

*Compound **4**.* Triethyl phosphite (1.2 mL, 7.0 mmol, 5.4 equiv) was added to **3** (0.53 g, 1.29 mmol, 1.0 equiv), and the mixture was stirred at 150 °C for 1 h. The reaction mixture was cooled to room temperature and directly submitted to flash chromatography (SiO_2, 5 × 15 cm, 100% ethyl acetate) to afford **4** (0.43 g, 0.54 g theoretical, 80%) as a colorless oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.20−4.05 (m, 4H, $CH_3C\mathit{H}_2OP$), 3.85−3.60 (m, 12H, CH_2OR), 3.54 (t, J = 5.6 Hz, 2H, $CH_2OTBDMS$), 2.15 (m, 2H, CH_2P), 1.31 (t, J = 6.0 Hz, 6H, $C\mathit{H}_3CH_2OP$), 0.88 (s, 9H, CH_3C), 0.0 (s, 6H, CH_3Si); MALDI-FTMS *m*/*z* 451.2257 ($C_{18}H_{41}O_7PSi + Na^+$ requires 451.2251).

*Compound **5**.* A solution of compound **4** (0.21 g, 0.5 mmol, 1.0 equiv) in CH_2Cl_2 (2.8 mL, 0.18 M) was treated with HF-pyridine (0.084 mL, ~0.84 mmol, ~1.7 equiv). The reaction was stirred at 25 °C for 30 min and then partitioned between ethyl acetate (100 mL) and water (100 mL). The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. Chromatography (SiO_2, 2 × 8 cm, 3−10% CH_3OH-CH_2Cl_2) afforded **5** (0.050 g, 0.28 g theoretical, 32.5%) as a clear oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.20−4.05 (m, 4H, $CH_3C\mathit{H}_2OP$), 3.80−3.55 (m, 14H, CH_2OR and CH_2OH), 2.15 (m, 2H, CH_2P), 1.31 (t, J = 6.0 Hz, 6H, $C\mathit{H}_3$-CH_2OP); MALDI−FTMS *m*/*z* 337.1377 ($C_{12}H_{27}O_7P + Na^+$ requires 337.1387).

*Compound **6**.* A solution of **5** (0.030 g, 0.096 mmol, 1.0 equiv) in DMF (0.28 mL, 0.34 M) was treated sequentially with *N*,*N*-disuccinimidyl carbonate (0.058 g, 0.22 mmol, 2.2 equiv) and triethylamine (0.035 μL, 0.25 mmol., 2.5 equiv). The reaction mixture was stirred at room temperature for 12 h and then partitioned between CH_2Cl_2 (100 mL) and H_2O (100 mL). The organic layer was washed with saturated aqueous NaCl (100 mL), dried (Na_2SO_4), and concentrated under reduced pressure. Chromatography (SiO_2, 2 × 8 cm, 1−10% CH_3OH-CH_2Cl_2) afforded **6** (0.035 g, 0.043 g theoretical, 81%) as a clear oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.45 [m, 2H, C*H*$_2$OC(O)OR], 4.20−4.05 (m, 4H, CH_3CH_2-OP), 3.80−3.55 (m, 12H, CH_2OR), 2.84 [s, 4H, $CH_2C(O)N$], 2.15 (m, 2H, CH_2P), 1.31 (t, J = 6.0 Hz, 6H, C*H*$_3$CH$_2$OP). MALDI−FTMS *m*/*z* 478.1456 ($C_{17}H_{30}NO_{11}P + Na^+$ requires 478.1449).

*Compound **7**.* A solution of **6** (0.020 g, 0.044 mmol, 1.0 equiv) in CH_2Cl_2 (0.14 mL, 0.40 M) was cooled to 0 °C and treated with oxalyl chloride (0.082 mL, 2 M in CH_2Cl_2, 0.164 mM, 3.7 equiv). The reaction mixture was allowed to warm to room temperature and stirred for 18 h. The reaction mixture was then concentrated under a stream of gaseous nitrogen and the remaining residue was treated with H_2O (0.1 mL) for 5 min. The solvent was evaporated under a stream of gaseous nitrogen and the remaining residue dried by vacuum to provide **7** (0.015 mg, 0.019 mg theoretical, 80%) as a clear oil/film: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.45 [m, 2H, C*H*$_2$OC(O)OR], 4.10 (m, 2H, CH_3CH_2OP), 3.80−3.55 (m, 12H, CH_2OR), 2.84 [s, 4H, $CH_2C(O)N$], 2.15 (m, 2H, CH_2P), 1.31 (t, J = 6.0 Hz, 3H, C*H*$_3$CH$_2$OP); MALDI−FTMS *m*/*z* 428.1320 ($C_{15}H_{26}NO_{11}P + H^+$ requires 428.1316).

*Compound **8**.* A solution of **7** (0.007 g, 0.016 mmol, 1.0 equiv) in CH_2Cl_2 (0.22 mL, 0.075 M) at −78 °C was treated with (diethylamino)sulfur trifluoride (DAST, 0.007 mL, 0.048 mmol, 3.0 equiv), and the reaction mixture was stirred for 10 min. The reaction mixture was then partitioned between ethyl acetate (100 mL) and H_2O (100 mL) and the organic layer was washed with saturated aqueous NaCl (100 mL), dried (Na_2SO_4), and concentrated under reduced pressure. Chromatography (SiO_2, Pasteur pipet, 100% ethyl acetate) afforded **8** (0.003 g, 0.007 g theoretical, 42%) as a clear oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.45 [m, 2H, C*H*$_2$-OC(O)OR], 4.27 (m, 2H, CH_3CH_2OP), 3.80−3.55 (m, 12H, CH_2OR), 2.84 [s, 4H, $CH_2C(O)N$], 2.32−2.26 (m, 2H, CH_2P), 1.31 (t, J = 6.0 Hz, 3H, C*H*$_3$CH$_2$OP).

FP-Peg-Biotin (9). A solution of **8** (0.003 g, 0.007 mmol, 1.0 equiv) in DMF (0.1 mL, 0.07 M) was added to 5-(biotinamido)-pentylamine (Pierce, 0.0035 g, 0.011 mmol, 1.5 equiv), and the reaction mixture was stirred for 4 h. The solvent was evaporated under a stream of gaseous nitrogen and the remaining residue was washed sequentially with diethyl ether and ethyl acetate, solubilized in a minimal volume of chloroform, transferred to a clean glass vial, and the solvent was evaporated. This process was repeated twice more to rid the desired biotinylated product of excess reagents and byproducts, affording **9** as a white film (0.0017 g, 0.0045 g theoretical, 38%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.04 (b s, 1H, NH), 5.77 (b s, 1H, NH), 5.24 (b s, 1H, NH), 5.09 (b s, 1H, NH), 4.51 (m, 1H), 4.40−4.21 [m, 5H, including C*H*$_2$OC(O)NR and CH_3CH_2OP], 3.90−3.55 (m, 12H, CH_2OR), 3.30−3.11 [m, 5H, including C*H*$_2$NHC(O)], 2.92 (dd, J = 4.9 and 12.9 Hz, 1H), 2.74 (d, J = 12.9 Hz, 1H), 2.35−2.18 (m, 4H, C*H*$_2$CONHR and C*H*$_2$P), 1.85−1.40 (m, 12H); 1.31 (t, J = 6.0 Hz, 3H, C*H*$_3$CH$_2$OP); MALDI-FTMS *m*/*z* 665.2742 ($C_{26}FH_{48}N_4O_9PS + Na^+$ requires 665.2756).

Tissue Sample Preparation. Soluble fractions of rat testis were prepared as described previously (*15*). Membrane fractions from rat tissues were prepared as follows. Rat tissue was Dounce-homogenized in 50 mM Tris-HCl buffer (pH 8.0) containing 0.32 M sucrose. The crude homogenate was centrifuged for 10 min at 1100*g*, and the supernatant then was centrifuged for 25 min at 22000*g* to provide a heavy membrane fraction. The heavy membrane fraction was resuspended in 50 mM Tris-HCl buffer (pH 8.0) containing 1 M NaCl and rotated for 60 min at 4 °C. After centrifugation for 25 min at 22000*g*, the resulting pellet was resuspended in 50 mM Tris-HCl buffer (pH 8.0) containing 1% Triton-X-100 at a final protein concentration of 5 mg/mL. The suspension was then rotated for 1 h at 4 °C and centrifuged at 22000*g* for 25 min. The supernatant from this spin was removed, constituting the solubilized membrane protein fraction. The solubilized membrane fraction was adjusted to 1 mg of protein/mL and then incubated for 1 h at 4 °C with one-tenth volume of avidin-agarose beads (Sigma) to deplete endogenous avidin-binding proteins. After a brief spin to pellet the avidin beads (2 min at 10000*g*), the solubilized protein fraction was removed and treated with biotinylated FPs as described below.

Reactions between Biotinylated FPs and Complex Proteomes. Standard conditions for FP-proteome reactions were as follows: protein (1 μg/μL) was treated with biotinylated FP (4 μM from a 100−200 μM stock in DMSO; final DMSO reaction concentration of 2−4%) for 1 h in 50 mM Tris·HCl, pH 8.0. Reactions were quenched with one volume of standard 2× SDS−PAGE loading buffer (reducing). Control reactions consisted of protein samples that were preheated at 80 °C for 5 min prior to treatment with biotinylated FP. Proteins that showed heat-sensitive FP reactivity were considered specific targets, while proteins showing heat-insensitive FP reactivity were considered nonspecific targets. pH-dependence studies were conducted using a reaction buffer of 50 mM Bis-Tris propane, 50 mM CAPS, 50 mM citrate, 150 mM NaCl. The pH was adjusted using NaOH or HCl.

Analysis of FP-Proteome Reactions. FP-labeled proteins were separated by SDS−PAGE and detected by blotting with avidin as described previously (*15*).

Fatty Acid Amide Hydrolase (FAAH) Catalytic Assays. FAAH catalytic activity was measured by following the conversion of ^{14}C-oleamide to ^{14}C-oleic acid as described previously (*33*).

Competition Assays between Noncovalent Serine Hydrolase Inhibitors and Biotinylated FPs. Solubilized brain membrane proteins (1 μg/μL) in reaction buffer (50 mM Tris·HCl, pH 8.0, 1.0% Triton) were treated sequentially with oleoyl trifluoromethyl ketone (OTFMK; final concentration of 1, 5, 50, or 200 μM) and biotinylated FP (final FP-biotin or FP-peg-biotin concentration of 5 μM), and the reaction mixtures were incubated at room temperature for 30 min. The reactions were then quenched with SDS−PAGE loading buffer and analyzed by SDS−PAGE-avidin blotting as described above. Both OTFMK and the biotinylated FPs were

added from concentrated stocks in DMSO to give a final DMSO concentration in the reactions of 5%. OTFMK was synthesized as described previously (*34*).

Avidin-Based Affinity Purification and Molecular Characterization of FP-Labeled Proteins. Protein samples from rat testis were incubated with biotinylated FPs under standard conditions (2 mL reactions), after which the reactions were passed over a PD-10 size exclusion chromatography column (Amersham-Pharmacia). Protein eluted from the PD-10 column was treated with SDS (to 0.5% w/v), heated, and diluted 2.5-fold prior to mixing with 100 μL of avidin-agarose beads (Sigma) for 1 h at room temperature. The avidin beads were washed twice with 50 mM Tris-HCl, 0.2% SDS and twice with 50 mM Tris-HCl. Elution of bound protein from the avidin beads was achieved by heating in one volume of 2× SDS−PAGE loading buffer. Avidin-enriched, FP-labeled proteins were separated on an SDS−PAGE gel, identified by staining with Coomassie blue, and excised from the gel with a razor blade. The excised protein bands were digested with trypsin and the resulting peptides analyzed by matrix assisted laser desorption (MALDI) mass spectrometry using a Voyager-Elite time-of-flight mass spectrometer with delayed extraction (PerSeptive Biosystems, Inc., Framingham, MA). The MALDI peptide data were used in MS-Fit searches of the ProteinProspector databases (peptide mass tolerance: ± 100 ppm; maximum one missed cleavage), which identified seven of the eight FP-labeled proteins (see Results for names of identified proteins). At least nine tryptic peptides were identified by MALDI for each of these proteins. Tryptic peptides from the eighth protein were examined by microcapillary HPLC-MS/MS using an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA) combined with a Finnigan LCQ mass spectrometer (Finnigan, San Jose, CA). Analysis of the tandem mass data from one peptide ($M + H^+ = 1099$ Da) provided the sequence GFVVAAIEHR, which was used in BLAST searches to identify the parent protein as a novel rat homologue of human and dog LDL-associated phospholipase A_2.

RESULTS

*Synthesis and Characterization of FP-Peg-Biotin (**9**).* A first generation serine hydrolase probe, FP-biotin, possessed a linear decamethylene chain connecting its FP reactive group to 5-(biotinamido)-pentylamine through an amide bond (*15*). We questioned whether the hydrophobic nature of FP-biotin's alkyl chain linker might hinder or prevent this agent from reacting with certain serine hydrolases, thereby leading to undesired false negatives (i.e., active serine hydrolases that remained unreactive toward FP-biotin). To address this issue, a variant of FP-biotin in which the agent's decamethylene chain was replaced by a more hydrophilic tetraethelyne glycol linker was synthesized as outlined in Scheme 1. Briefly, tetraethyleneglycol (**1**) was monosilyated to provide compound **2**, which following conversion to alkyl iodide **3**, was reacted with triethyl phosphite to give phosphonate **4**. Desilyation of **4** with HF-pyridine, followed by treatment with *N*,*N*-disuccimidyl carbonate, provided NHS-carbonate **6**. Compound **6** was converted to monoethoxy phosphonate acid **7** by treatment with oxalyl chloride followed by hydrolytic workup. Compound **7** was treated with DAST to give fluorophosphonate **8**, which was then coupled to 5-(biotinamido) pentylamine (Pierce) to provide FP-peg-biotin (**9**).

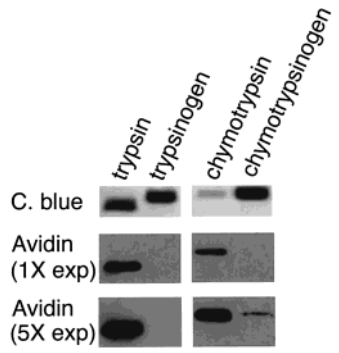


FIGURE 1: FP-peg-biotin labels serine proteases but not their respective zymogens. Samples of each protein (100 nM, Sigma) were treated with FP-peg-biotin (4 μM) for 1 h (50 mM Tris, pH 7.2), quenched with 2× SDS−PAGE loading buffer (reducing), and analyzed by SDS−PAGE and blotting with avidin (45 ng protein/lane, bottom two panels). 50× protein stocks were also run on an SDS−PAGE gel and stained with Coomassie blue (2.2 μg/lane, top panels). Note that the weak avidin signal seen in the chymotrypsinogen sample upon longer exposures may represent FP-peg-biotin reactivity with trace amounts of contaminating chymotrypsin, which is reported to compose up to 2% of the purchased proenzyme.

To test whether FP-peg-biotin reacted with serine hydrolases in an activity-dependent manner, the agent was incubated with equal amounts of two serine proteases (trypsin and chymotrypsin) and their respective zymogens (trypsinogen and chymotrypsinogen). The products of each reaction were compared by SDS−PAGE and blotting with avidin (Figure 1). FP-peg-biotin strongly labeled both trypsin and chymotrypsin, but exhibited little or no reactivity with their corresponding proenzymes. Protein staining revealed that a significant fraction of the chymotrypsin sample had been degraded during the course of setting up the reaction. Nonetheless, FP-peg-biotin still showed a much stronger labeling intensity with the chymotrypsin sample than with its chymotrysinogen counterpart, despite the substantially lower quantity of protein present in the former reaction. Indeed, the low level of FP-peg-biotin labeling observed in the chymotrypsinogen sample (seen only in the 5× exposure panel) may have actually represented reactivity with trace amounts of chymotrypsin rather than with the zymogen itself, as the purchased proenzyme is reported to possess up to 2% active protease.

Comparing the Serine Hydrolase Activity Profiles Generated with FP-Biotin and FP-Peg-Biotin. Soluble fractions of rat testis (1 μg/μL) were treated with either FP-biotin or FP-peg-biotin (4 μM) for 1 h at room temperature (50 mM Tris, pH 7.2, 150 mM NaCl) and the labeled serine hydrolase activities detected by SDS−PAGE and blotting with avidin. Control reactions in which the proteome was heat-denatured prior to treatment with biotinylated FPs were also analyzed to distinguish specific (heat-sensitive) from nonspecific (heat-insensitive) protein reactivity. The serine hydrolase activity profiles generated with each biotinylated FP were strikingly similar to one another with a single notable exception (Figure 2, panel A). A pair of 48-kDa serine hydrolase activities was strongly labeled by FP-peg-biotin, but displayed very low reactivity with FP-biotin (arrowhead). FP-proteome reactions conducted for longer times (2 h to overnight) increased the

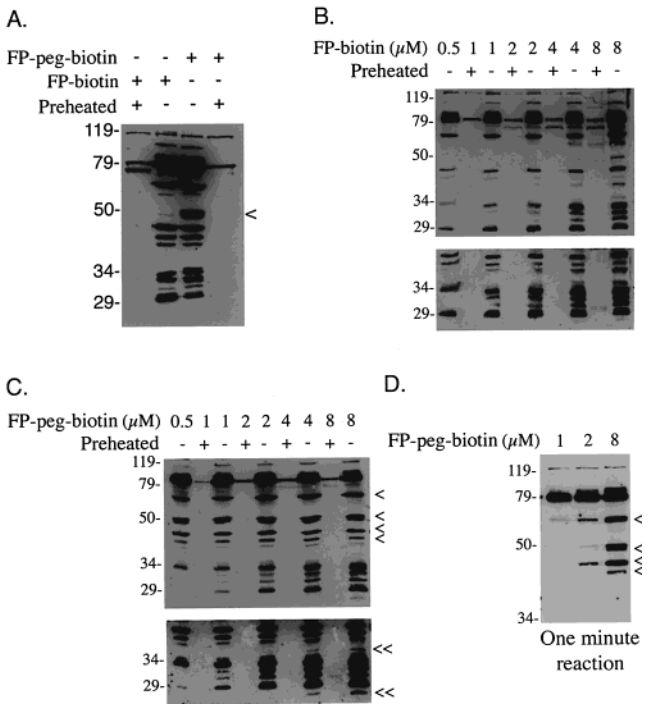


FIGURE 2: Comparing the proteome reactivities of FP-biotin and FP-peg-biotin. For all of the experiments depicted in this figure, protein samples represent soluble fractions of rat testis (1 μg of protein/μL). (A) Protein samples were treated with 4 μM of either FP-biotin or FP-peg-biotin for 1 h (50 mM Tris, pH 7.2) and analyzed by SDS−PAGE and blotting with avidin. A pair of 48-kDa serine hydrolases that were robustly labeled by FP-peg-biotin, but not FP-biotin are highlighted (arrowhead). (B−D) Concentration dependence of FP−proteome reactions. Protein samples were treated with 0.5, 1, 2, 4, or 8 μM of either FP-biotin (B) or FP-peg-biotin (C) for 1 h (50 mM Tris, pH 7.2) and analyzed by SDS−PAGE and blotting with avidin. The upper and lower panels represent 1 and 10 min film exposures, respectively. Several serine hydrolases appearing to show no FP-peg-biotin concentration dependence in a 1 h reaction (C, arrowheads), exhibited strong probe concentration dependence in a 1-min reaction (D, arrowheads).

labeling intensity of some serine hydrolases, but did not result in the detection of any new heat-sensitive protein reactivities (data not shown). Therefore, 1 h FP-proteome reactions provided profiles that were considered to represent "maximal coverage" of serine hydrolase activities, and unless otherwise noted, subsequently described reactions were conducted for this length of time.

Optimizing the Specific Reactivity of Biotinylated FPs. The similar maximal coverage serine hydrolase activity profiles generated with FP-biotin and FP-peg-biotin indicated that the chemical nature of their respective linkers was not a major factor that determined whether these agents could react with most serine hydrolases. However, additional parameters, like probe concentration and pH, were suspected to substantially influence the reactivity of biotinylated FPs with certain serine hydrolases, potentially complicating the consolidated detection of active members of this enzyme family. Accordingly, optimal reaction conditions were sought for the analysis of serine hydrolase activities in complex proteomes. Optimal conditions were defined as those that maximized the ratio of a biotinylated FP's specific (heat-sensitive) to nonspecific (heat-insensitive) proteome reactivity.

Optimizing the Specific Reactivity of Biotinylated FPs: The Probe Concentration Dependence of FP-Proteome Reactions. Samples of the rat testis proteome were treated for 1 h with either FP-biotin or FP-peg-biotin over a probe concentration range of 0.5−8.0 μM (Figure 2, panels B and C). Most FP-biotin-labeled proteins displayed enhanced signal intensities with increasing amounts of probe, indicating that their reactivities were not saturated at low micromolar concentrations of FP-biotin. In contrast, several of the FP-peg-biotin-labeled proteins showed no detectable change in their signal intensities with increasing concentrations of probe (Figure 2, panel C, arrowheads). These enzymes had either reacted to completion, or were saturated in their rates of labeling at all of the FP-peg-biotin concentrations tested. Kinetic experiments supported the former explanation, as clear concentration-dependent labeling for all of these proteins could be observed in reactions conducted for a shorter time (1 min; Figure 2, panel D, arrowheads).

Notably, in reactions conducted for 1 h with concentrations of 4−8 μM FP-peg-biotin, at least 18 distinct serine hydrolase activities could be resolved on a single lane of a one-dimensional SDS−PAGE gel (Figure 2, panel C). Several of these serine hydrolases could not be detected at lower concentrations of probe (double arrowhead). An even higher concentration of FP-peg-biotin (16 μM) produced a qualitatively similar serine hydrolase activity profile to that observed with 4 and 8 μM probe (i.e., no new heat-sensitive protein reactivities were detected at 16 μM) but also generated a high level of nonspecific protein reactivity (data not shown). Therefore, it was concluded that biotinylated FPs displayed optimal ratios of specific to nonspecific proteome reactivity at concentrations ranging from 4 to 8 μM.

Optimizing the Specific Reactivity of Biotinylated FPs: The pH-Dependence of FP−Proteome Reactions. A single mixed buffer assay was used to evaluate the pH-dependence of FP-proteome reactions over a pH range of 6.0−9.0 (conditions: 1 μg/μL soluble testis protein, 4 μM biotinylated FP, 50 mM Bis-Tris propane, 50 mM CAPS, 50 mM citrate, 150 mM NaCl; 1 h reaction). Several forms of pH-dependence were observed among the FP-labeled proteins, with some serine hydrolases exhibiting an optimal FP reactivity at pH 8.0 (Figure 3, triple arrowheads) and others showing an FP reactivity that continued to increase in intensity as the pH was raised to 9.0 (double arrowheads). Although multiple serine hydrolases appeared to show "pH-independent" FP reactivities, kinetic analyses indicated that most of these enzymes had labeled to completion during the time course of the reactions (see above). Thus, these enzymes likely react with FPs in a pH-dependent manner that might be visualized by modifying the parameters of the reaction to slow their rates of labeling (e.g., lowering the probe concentration and/or reducing the incubation time). Finally, nonspecific FP reactivity increased significantly at pH 9.0, with several labeled proteins appearing in the preheated control lanes (single arrowheads). Coomassie blue staining revealed that these labeled proteins were all high abundance constituents of the testis proteome (data not shown), supporting the notion that heat-insensitive labeling represents a nonspecific form of FP reactivity. Considering further that the majority of serine hydrolases displayed similar (or greater) FP reactivities at pH 8.0 relative to pH 9.0, the former pH appears better suited for the functional analysis of this enzyme family in complex proteomes.

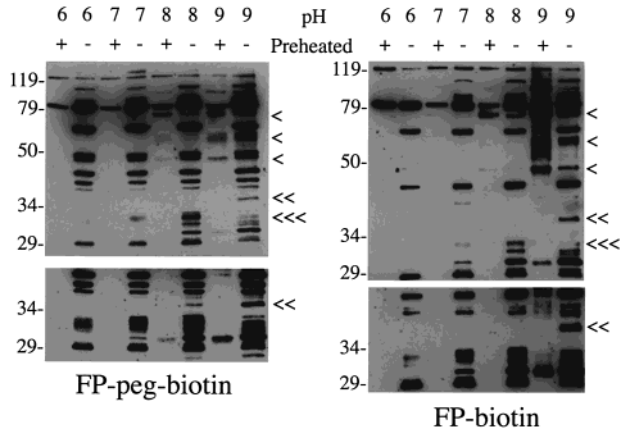


FIGURE 3: pH-dependence of FP−proteome reactions. Protein samples were treated with either FP-peg-biotin (left panel) or FP-biotin (right panel) for 1 h (50 mM Tris·50 mM CAPS·50 mM sodium citrate) at pH 6.0, 7.0, 8.0, or 9.0 and analyzed by SDS−PAGE and blotting with avidin. Serine hydrolases displaying pH optima of 8 and 9 are highlighted by triple and double arrowheads, respectively. Proteins exhibiting heat-insensitive FP-reactivity at pH 9 are highlighted by single arrowheads. The upper and lower panels represent 1 and 10 min film exposures, respectively.

Kinetic Analysis of FP-Proteome Reactions. Although single time point measurements offer a simple and rapid means to obtain a general profile of the active serine hydrolases present in complex proteomes, kinetic analyses are required to decipher more intricate changes in enzyme activity. For example, alterations in serine hydrolase activity that take place in the absence of changes in enzyme abundance may remain undetected with biotinylated FPs unless rates of reactivity are measured. To examine the kinetics of FP-proteome reactions, rat brain membrane fractions were treated with biotinylated FPs and the time course of protein labeling followed by SDS−PAGE and blotting with avidin. The goal of these studies was to determine whether serine hydrolases exhibited rates of FP reactivity that could be experimentally monitored in complex proteomes, and if so, whether these rates differed significantly with FP-biotin and FP-peg-biotin. Additionally, by selecting a membrane proteome for analysis, the ability of biotinylated FPs to profile membrane-associated (as well as soluble) serine hydrolases could be directly tested.

Initially, brain membrane proteins were treated with FP-biotin both prior to and after solubilization with Triton X-100 to examine the effects of detergent on the FP−membrane proteome reaction. Similar serine hydrolase activity profiles were observed with membrane-associated and Triton-solubilized brain protein samples (Figure 4), indicating that detergent solubilization maintained most of the membrane-bound serine hydrolases in a catalytically active state. Therefore, subsequent labeling experiments were conducted with Triton-solubilized brain membrane proteins.

Most brain membrane serine hydrolases exhibited rates of FP reactivity that could be monitored over a time course of 1−60 min under the following set of reaction conditions: 4 μM biotinylated FP, 1 μg/μL protein, 50 mM Tris buffer, pH 8.0, with 0.2% Triton X-100. Interestingly, examples were observed for each of the three potential types of serine hydrolase reactivity profiles: (i) enzymes that reacted with FP-biotin at faster rates than with FP-peg-biotin (Figure 5, panel A, single arrowhead), (ii) enzymes that reacted with

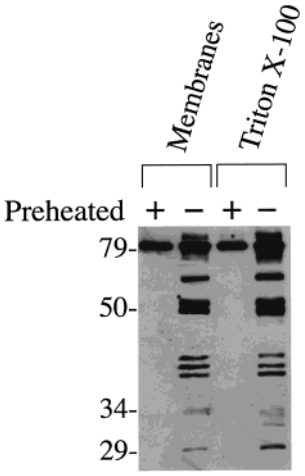


FIGURE 4: The serine hydrolase activity profile of Triton-solubilized brain membranes is similar to that of unsolubilized brain membranes (reaction conditions: 1 μg/μL protein, 2 μM FP-biotin, 30 min reaction, 50 mM Tris, pH 8.0, with or without 0.2% Triton X-100).

FP-peg-biotin at faster rates than with FP-biotin (double arrowheads), and (iii) enzymes that reacted with FP-biotin and FP-peg-biotin at similar rates (triple arrowheads). In particular, a 65-kDa serine hydrolase activity was apparently labeled to completion with FP-biotin within one minute (Figure 5, panel A, left panel), but reacted at a much slower rate with FP-peg-biotin (its signal intensity still increasing from 30 to 60 min; Figure 5, panel A, right panel). We suspected that this serine hydrolase might represent fatty acid amide hydrolase (FAAH) (*20*), a brain integral membrane enzyme that displays a strong preference for long aliphatic amide substrates (Patricelli, M. P., and Cravatt, B. F., manuscript in preparation). To test this premise, we treated brain membrane proteins with each biotinylated FP and measured FAAH catalytic activity at two time points during the reaction. No FAAH activity could be detected in the FP-biotin treated samples after incubations for either 10 or 60 min. In contrast, significant FAAH activity was observed in the FP-peg-biotin treated samples at both time points, with the 10 and 60 min incubations displaying 60 and 30% FAAH activity, respectively (relative to an untreated control sample). Thus, FAAH's catalytic activity was inhibited at a much faster rate by FP-biotin than FP-peg-biotin, consistent with the relative rates of FAAH labeling by these two probes as detected in avidin blotting experiments (Figure 5, panel A).

Considering that several brain membrane serine hydrolases reacted preferentially with one biotinylated FP over the other, we speculated that by treating a single proteome with a mixture of both probes, a more complete serine hydrolase activity profile might be obtained. In support of this notion, treating brain membrane proteins with 2 μM each of FP-biotin and FP-peg-biotin provided a serine hydrolase activity profile that resembled closely the predicted merger of profiles generated with each FP individually (Figure 5, panel B). These data indicate that adding multiple activity-based probes to a single proteome ("multiplexing") can enhance the coverage of active enzymes present in that sample.

Comparing the Membrane-Associated Serine Hydrolase Activity Profiles from Different Rat Tissues. Intrigued by the large number of serine hydrolase activities associated with brain membranes, we compared the serine hydrolase activity profiles of membrane fractions from a panel of rat tissues.

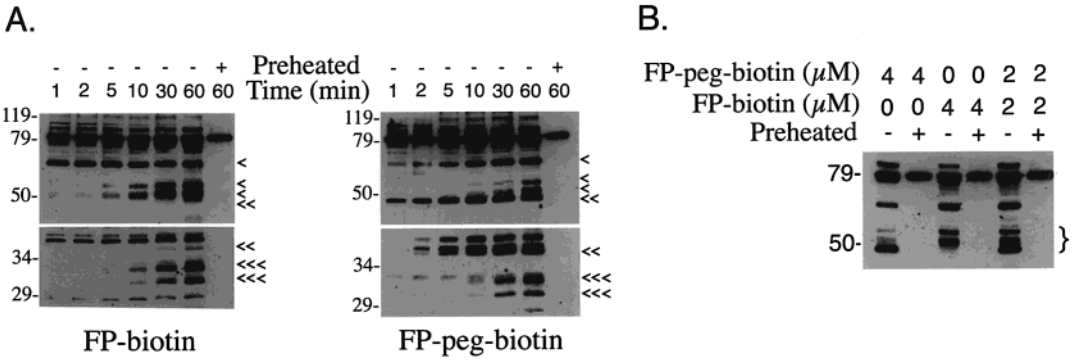


FIGURE 5: (A) Kinetic analysis of FP−proteome reactions. Triton-solubilized brain membranes (1 μg of protein/μL) were treated with 4 μM of either FP-biotin (left panel) or FP-peg-biotin (right panel) for the indicated reaction times, after which the assays were quenched with 2× SDS−PAGE loading buffer and analyzed by SDS−PAGE-avidin blotting. Serine hydrolase activities that reacted at a faster rate with FP-biotin or FP-peg-biotin are highlighted (single and double arrowheads, respectively), as are enzymes that reacted at equivalent rates with both probes (triple arrowheads). The upper and lower panels represent 1 and 5 min film exposures, respectively. (B) Multiplexing of biotinylated FPs enhances coverage of active serine hydrolases in complex proteomes. Solubilized brain membrane proteins were treated with the indicated concentrations of biotinylated FPs (30 min reaction). Highlighted are three serine hydrolases that are collectively visualized more clearly in an FP-proteome reaction containing a mixture of FP-biotin and FP-peg-biotin than in reactions conducted with each probe alone (bracket).

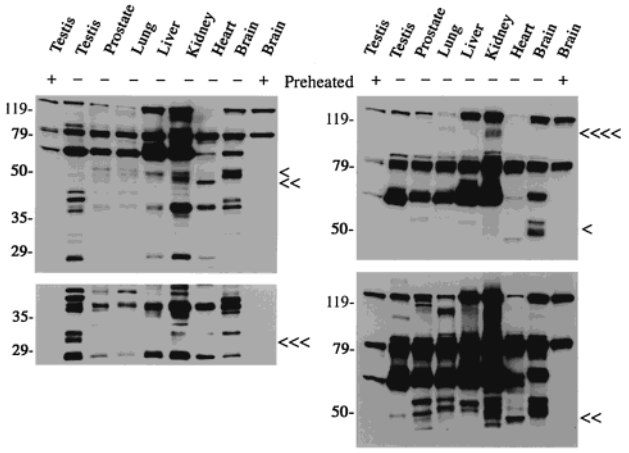


FIGURE 6: A comparison of membrane-associated serine hydrolase activities expressed in a panel of rat tissues. Protein samples (1 μg/μL) were treated with FP-biotin (4 μM) for 1 h (50 mM Tris, pH 8.0, 0.2% Triton), quenched with 2× SDS−PAGE loading buffer, and analyzed by SDS−PAGE/avidin blotting on either a 10% (left panels) or 8% (right panels) polyacrylamide gel. Highlighted are brain-enriched (single arrowheads), heart-enriched (double arrowheads), testis-enriched (triple arrowhead), and kidney-enriched (quadruple arrowhead) serine hydrolase activities. The upper and lower panels represent 0.5 and 2 min film exposures, respectively. The higher abundance serine hydrolase activities can be more effectively compared in the upper panels (0.5 min exposures), while the lower abundance serine hydrolase activities can more clearly be seen in the lower panels (2 min exposures).

Each membrane fraction was first washed with 1 M NaCl prior to solubilzation of its protein content with Triton X-100. This protocol was selected to enrich for integral membrane proteins, a class of proteins notoriously resistant to analysis by standard proteomics methods (*12*). Interestingly, each of the tissues examined possessed a unique and complex profile of membrane-associated serine hydrolase activities (Figure 6). Notably, a set of three 48−52 kDa serine hydrolase activities were enriched in brain membranes (single arrowhead). Likewise, heart-enriched, testis-enriched, and kidney-enriched serine hydrolase activities were also observed (double, triple, and quadruple arrowheads, respectively).

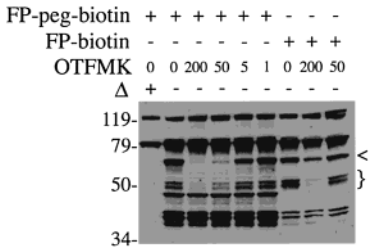


FIGURE 7: Evaluating the target selectivity of a reversible serine hydrolase inhibitor in complex proteomes. Competition reactions between varying concentrations of oleoyl trifluoromethyl ketone (OTFMK) and biotinylated FPs identifies several brain membrane serine hydrolases targeted by OTFMK. The arrowhead highlights the brain enzyme fatty acid amide hydrolase (FAAH), a known OTFMK target, and the bracket highlights two additional brain membrane serine hydrolases sensitive to OTFMK.

Evaluating the Target Selectivity of Reversible Serine Hydrolase Inhibitors in Complex Proteomes. Biotinylated FPs not only distinguish active serine hydrolases from zymogens (Figure 1), but also from inhibitor-bound enzymes (*15*). Thus, these chemical probes might serve as useful agents for determining the target selectivity of serine hydrolase inhibitors directly in complex proteomes, an objective of particular importance for reversible inhibitors, which often possess endogenous protein targets that are challenging to experimentally identify. With these issues in mind, each biotinylated FP was added to a brain membrane proteome treated with varying concentrations of oleoyl trifluoromethyl ketone (OTFMK), a previously described reversible FAAH inhibitor (*34*). After a 30-min incubation, the reactions were quenched and analyzed by SDS−PAGE and blotting with avidin. Interestingly, several serine hydrolases displayed reduced FP-reactivity in the presence of increasing concentrations of OTFMK (Figure 7), indicating that this electrophilic ketone was not only an effective inhibitor of FAAH, but of other brain membrane serine hydrolases as well. Film densitometry was used to estimate OTFMK's potency as an inhibitor of each of its serine hydrolase targets. A pair of 50 kDa serine hydrolases displayed approximately 40 and 80% reductions in both their FP-biotin and FP-peg-biotin reactivities in the presence of 50 and 200 μM OTFMK, respectively (bracket). In contrast,

FAAH's FP-peg-biotin reactivity was reduced by approximately 35, 95, and 99% in the presence of 5, 50, and 200 μM OTFMK, respectively (arrowhead). These data reveal that OTFMK displays a relatively modest (approximately 1 order of magnitude) selectivity for FAAH among other brain membrane serine hydrolases.

Although both 50 and 200 μM concentrations of OTFMK blocked at least 95% of FAAH's FP-peg-biotin reactivity, this enzyme's FP-biotin reactivity was only weakly affected (~20% reduction) and partially affected (~75% reduction) by 50 and 200 μM OTFMK, respectively (Figure 7). These apparently conflicting data can be understood by taking into account the vastly different rates at which FAAH reacted with the two biotinylated FPs. Indeed, FAAH was labeled by FP-biotin at a rate that was too fast to monitor under the reaction conditions employed (Figure 5, panel A, left panel). In such a case, the binding of a reversible inhibitor will only be detected if the inhibitor reduces its protein target's rate of FP reactivity to the extent that the protein no longer labels to completion during the time course of the reaction. Thus, FAAH's sensitivity to OTFMK was likely underestimated in the 30 min FP-biotin competition assay. On the other hand, FP-peg-biotin labeled FAAH at a rate slow enough to be followed over a 60-min time course (Figure 5, panel A, right panel), and therefore reactions conducted with this probe should have provided a more accurate assessment of the sensitivity of FAAH to competitive active site-directed agents. In support of this notion, OTFMK's IC_{50} value for inhibiting FAAH's catalytic activity in the brain solubilized membrane preparation was determined to be 5 μM, a value similar to that predicted from competition assays between this inhibitor and FP-peg-biotin (~35% reduction in FP-peg-biotin labeling in the presence of 5 μM OTFMK). Collectively, these results demonstrate that biotinylated FPs can, in cases where they display discernible labeling kinetics, identify the serine hydrolase targets of reversible inhibitors directly in complex proteomes.

Affinity Isolation and Molecular Characterization of FP-Labeled Serine Hyrdrolases. For activity-based probes such as the biotinylated FPs to be of lasting use to proteomics research, these reagents must not only serve as tools for protein detection, but also for protein isolation and identification. In the course of attempting to affinity purify FP-biotinylated proteins by avidin agarose chromatography, we noted that several labeled proteins failed to bind the avidin matrix in their native state (Figure 8, panel A, arrowheads). However, if protein samples were denatured prior to treatment with avidin beads, efficient binding of all of the biotinylated proteins was achieved. Subsequent washing of the beads and elution with SDS−PAGE loading buffer provided a sample greatly enriched for biotinylated proteins (Figure 8, panel A, right panel). The elution sample was run on an SDS−PAGE gel and the biotinylated proteins were excised from the gel and digested with trypsin, and the resulting peptide mixtures were analyzed by MALDI mass spectrometry. This one-step isolation method identified seven of the labeled serine hydrolases present in a soluble rat testis proteome as acylpeptide hydrolase (82 kDa, accession #CAA33040), prolyl oligopeptidase (80 kDa, BAA25544), carboxylesterase 1 (80 kDa, accession #JX0054), carboxylesterase 10 (60 kDa, accession #P16303), long chain acyl CoA hydrolase 2 (48 kDa, accession #088267), platelet-activating factor (PAF) acetylhydrolase α1 subunit (32 kDa, accession #NP_032802), and PAF acetylhydrolase α2 subunit (30 kDa, accession #035264) (Figure 8, panel B). An additional 45-kDa serine hydrolase activity provided a MALDI tryptic peptide map that did not match those of any proteins in the public databases. Analysis of the electrospray MS fragmentation pattern of one of the tryptic peptides from this protein ($M + H^+ = 1099$ Da) provided the following sequence information: GFVVAAIEHR. BLAST database searches with this peptide sequence identified 80 and 90% identical sequences in the human and dog versions, respectively, of a 45-kDa serine hydrolase referred to as plasma PAF acetylhydrolase or LDL-associated phospholipase A2 (human protein accession #AAB04170.1). Thus, the isolated 45-kDa FP-biotin-reactive testis protein likely represents a previously uncharacterized rodent member of this subclass of serine hydrolases.

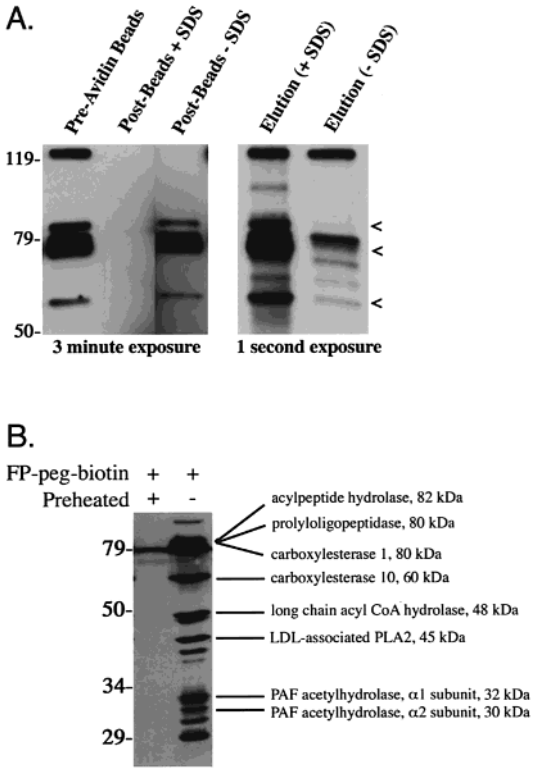


FIGURE 8: Affinity isolation and molecular characterization of FP-labeled serine hydrolase activities. (A) Avidin-agarose beads effectively bind denatured, but not native FP-labeled proteins. A sample of FP-biotin-labeled proteins (Pre-Avidin Beads) was mixed with avidin agarose beads with or without a predenaturation step (heating in 1% SDS for 10 min at 85 °C) for 1 h at room temperature. Visualization of the supernatants by SDS−PAGE/avidin blotting (Post-Beads) identified several proteins (arrowheads) that only bound the avidin beads in the SDS-treated sample (+SDS) and were strongly enriched in the corresponding elution fraction (Elution) relative to an untreated sample (−SDS). Note that the left panel represents a 3-min exposure, while the right panel represents a 1-s exposure. The different exposure times reflect the high degree of concentration of FP-biotin-labeled proteins achieved by this method. (B) Molecular identification FP-biotin-labeled proteins. Eight of the avidin-enriched FP-labeled proteins were excised from an SDS−PAGE gel and digested with trypsin, and the resulting peptides were analyzed by MALDI and/or electrospray mass spectrometry. Mass information identified all of these proteins as serine hydrolases (see text for GenBank accession numbers).

DISCUSSION

The stated goal of profiling with biotinylated fluorophosphonates (FPs) the complete repertoire of active serine hydrolases present in a given proteome mandates experimental inquiry into the range of potential biochemical properties exhibited by this large class of enzymes. For example, variability among serine hydrolases in terms of their substrate binding sites, pH-optima, and/or inherent catalytic power could compromise efforts to characterize their activities collectively using a single chemical probe and/or set of reaction conditions. Additionally, the utility of FPs as profiling agents may depend to a significant degree on the ratio of their specific versus nonspecific proteome reactivities, a relationship that in turn might vary considerably with the reaction conditions employed. In this study, we have explored the FP reactivity of complex proteomes under a variety of experimental conditions, permitting the identification of parameters that facilitate both the simultaneous detection and functional characterization of numerous members of the serine hydrolase superfamily.

Of primary concern in our initial investigations with FP-biotin was the potential bias that this agent might show toward binding (and, as a consequence, reacting with) serine hydrolases that possess hydrophobic active sites. Therefore, a variant of FP-biotin was synthesized in which the agent's decamethylene linker was replaced with a more hydrophilic tetraethylene glycol tether. The proteome reactivity of this second generation probe, FP-peg-biotin, was compared to that of FP-biotin under a variety of experimental conditions. Interestingly, both FP agents generated similar "maximal coverage" serine hydrolase activity profiles (defined for a given proteome, as profiles displaying the largest number of detectable heat-sensitive protein targets), indicating that the chemical nature of their respective linkers was not a determining factor for absolute reactivity with most serine hydrolases. However, one notable exception was observed, as a pair of 50-kDa serine hydrolase activities was identified that predominantly reacted with FP-peg-biotin (Figure 2, panel A). Somewhat surprisingly, we identified one of these serine hydrolases as long chain acyl CoA hydrolase 2 (LACH2), an enzyme that based on its proposed endogenous substrates (long chain fatty acyl CoAs; *35*) might have been expected to show greater reactivity with the more hydrophobic probe, FP-biotin. LACH-2's selective reactivity with FP-peg-biotin suggests that this recently characterized enzyme might possess an active site more suited for binding hydrophilic substrates/inhibitors than initially supposed.

FP-proteome reactions were examined for their pH and probe concentration dependencies, permitting the identification of conditions optimally suited for the global analysis of serine hydrolase activities in complex proteomes: pH $\sim$ 8, FP concentration $=$ 4$-$8 μM. These empirically derived conditions resulted in the highest ratios of specific (heat-sensitive) to nonspecific (heat-insensitive) protein reactivity, leading to the detection in a soluble rat testis proteome of at least 18 FP-peg-biotin-labeled proteins (as visualized in a single lane of a one-dimensional SDS$-$PAGE gel by blotting with avidin; Figure 2, panel C). Avidin-based affinity purification of these biotinylated proteins, followed by analysis of their respective tryptic peptide maps with mass spectrometry, identified eight of the labeled proteins, all of which represented serine hydrolases: two peptidases, two esterases, one fatty acyl coA hydrolase, and three lipases. These data support the notion that FPs are highly selective affinity agents for the serine hydrolase class of enzymes.

Biotinylated FPs were also effective at labeling membrane-associated serine hydrolase activities, generating in these fractions profiles of substantial complexity and diversity. Indeed, the sum total of membrane-associated serine hydrolase activities identified in a panel of rat tissues rivaled the number of serine hydrolases observed in the soluble fractions from these same tissues (*15*). The ability of chemical probes such as the biotinylated FPs to profile membrane, as well as soluble protein activities distinguishes these reagents from more traditional proteomics tools, which often encounter difficulty analyzing membrane-associated proteins (*12*).

Serine hydrolases are subjected to several forms of posttranslational regulation in vivo. For example, most serine proteases are produced as catalytically inactive precursor proteins (zymogens) that must be processed to gain proteolytic function (*27*). Additionally, once activated, serine proteases are targeted by large families of endogenous protein inhibitors, including serpins (*28*) and Kunitz domain proteins (*29*). Such posttranslational mechanisms for regulating serine hydrolase activity complicate the functional analysis of this enzyme family by standard genomics and proteomics methods, which focus on measuring changes in transcript and protein abundance, respectively. In contrast, biotinylated FPs react with serine hydrolases in an activity-dependent manner, labeling active enzymes, but not their zymogen (Figure 1) or inhibitor-bound forms (Figure 7). Thus, proteomics investigations employing biotinylated FPs are especially well qualified to identify changes directly in serine hydrolase activity, even in cases where enzyme abundance remains constant.

The range and diversity of endogenous functions played by serine hydrolases in both health and disease have made these enzymes attractive targets for drug discovery (*36*$-$*38*). Medicinal chemistry efforts directed at serine hydrolases have produced many types of potent inhibitors, often possessing an electrophilic carbonyl as an active site-directed binding group [e.g., α-keto amides (*34*, *39*), trifluoromethyl ketones (*39*, *40*), lactones (*41*), α-ketoheterocycles (*42*, *43*), etc.]. Although such electrophilic substituents contribute significantly to inhibitor potency, they may also compromise inhibitor selectivity among members of the serine hydrolase class. Accordingly, methods are needed to assess the target selectivity of such "mechanism-based" reversible serine hydrolase inhibitors, a particularly daunting task when the immense size and diversity of this enzyme family are taken into consideration. The broad spectrum reactivity of biotinylated FPs with members of the serine hydrolase family may make these agents well suited for evaluating the target selectivity of reversible serine hydrolase inhibitors directly in complex proteomes. In this study, biotinylated FPs were used to examine the selectivity of a previously described reversible FAAH inhibitor, oleoyl trifluoromethyl ketone (OTFMK; *34*). Fatty acyl trifluoromethyl ketones have been characterized as moderately potent FAAH inhibitors (IC_{50} values of 0.5$-$5 μM; *40*, *44*, *45*), suggesting that these chemical agents might be useful for studying FAAH's function in vivo. However, competition assays with bioti-

nylated FPs identified multiple brain membrane serine hydrolases in addition to FAAH that were targeted by OTFMK, revealing that this inhibitor exhibits relatively poor selectivity among members of the serine hydrolase family.

This initial study indicates that biotinylated FPs can serve as a valuable first screen to test whether reversible serine hydrolase inhibitors exhibit high selectivity for their intended targets within this large enzyme class. Still, it is important to qualify that an FP-based assay will not reveal whether a given inhibitor has additional protein targets outside of the serine hydrolase superfamily. Hopefully, as the number of activity-based probes that target additional enzyme classes continues to grow (*14*, *46*−*48*), a menu of chemical reagents will become available that can more comprehensively assess the target selectivity of inhibitors not only within a given enzyme family, but also among different classes of enzymes.

Notably, the interaction of FAAH with OTFMK was detected in competition reactions with FP-peg-biotin, but not in equivalent assays with FP-biotin. These apparently conflicting data can be rationalized as follows. Kinetic analyses revealed that FAAH reacted to apparent completion with FP-biotin within one minute, while the enzyme was labeled by FP-peg-biotin at a much slower rate (only ∼70% inhibition of FAAH activity was achieved at 1 h). Thus, the FAAH−FP-biotin reaction occurred too rapidly to permit detection of competitive binding by the reversible OTFMK inhibitor. Collectively, these results emphasize the importance of determining in a given FP-proteome reaction which serine hydrolases exhibit labeling rates that can be experimentally followed, as only these enzymes are suitable for more elaborate forms of activity-based analysis (i.e., for detecting relative as well as absolute changes in catalytic activity). Notably, because FP-biotin and FP-peg-biotin exhibited complimentary labeling kinetics with many serine hydrolases, changes in enzyme activity that cannot be observed with one probe may prove detectable with the other (as was the case for FAAH).

In summary, the generation and characterization of biotinylated FPs bearing chemically complimentary linkers has significantly expanded the number of serine hydrolases accessible to activity-based proteomics investigations. Biotinylated FPs were used to rapidly detect, isolate, and identify several serine hydrolase activities directly in complex proteomes, facilitating the functional characterization of both soluble and membrane-associated members of this enzyme superfamily. Considering that biotinylated FPs profile such a large and diverse array of serine hydrolases in an activity-dependent manner, these probes should furnish insights into the expression and function of this class of enzymes that are both complimentary, and in many cases, superior to those provided by more standard, abundance-based genomics and proteomics methods.

ACKNOWLEDGMENT

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